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A SIMPLE METHOD FOR MEASURING CARBON DIOXIDE PRODUCED BY SMALL ORGANISMS.¹

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The suitable methods available at the present time for measuring the carbon dioxide produced by single cells such as eggs and small organisms weighing not over one or two grams are limited in number and usually only suitable for special types of material. Among the best methods for measuring or comparing small quantities of carbon dioxide produced by small organisms are those by Thunberg ('05) as modified by Winterstein ('13), and Krogh ('16). These are in turn based on the older and well known methods of Pettenkoffer and Petterson. Another method is that used by Warburg ('09) in his studies on sea urchin eggs and a more recent and apparently extremely sensitive method for the detection and estimation of very small quantities of CO₂ is that of Tashiro ('17). All of these methods may have their own advantages, depending upon the material and the nature of the problem. However, if many separate determinations of CO₂ are to be made at the same time, the apparatus must be duplicated or if not, the complexity of the procedure is increased greatly and thus becomes practically impossible.

In attempting to devise some simple method for measuring at the same time the small amounts of CO₂ produced by separate samples of *Paramecium*, or small invertebrates weighing one gram or less, the following procedure was adopted. Fig. 1 and the photograph, Fig. 2, show the arrangement. Two bottles of about two liters capacity, one containing $n/100$ HCl and the other a solution of Ba(OH)₂ of any concentration between $n/75$ and $n/150$, are provided with soda lime tubes and burettes. The solutions should be made up with CO₂ free distilled water as described in Treadwell and Hall ('15). A bottle of about 100 c.c. capacity, has a large glass stopper which fits the bottle very accurately and from which is suspended a small dish by

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means of the hook and wire. The stender dish, Fig. 1, *d*, may either be suspended as shown or held rigidly by an appropriately shaped clamp of wire fixed to the stopper with cement.

The procedure in brief is as follows: One to five cubic centimeters of a suspension of *Paramecium* which have previously been gradually transferred to pure tap water from native hay infusion are placed in the dish, Fig. 1, *d*. Ten or fifteen cubic centimeters of the $n/100$ $\text{Ba}(\text{OH})_2$ solution are run into the bottle

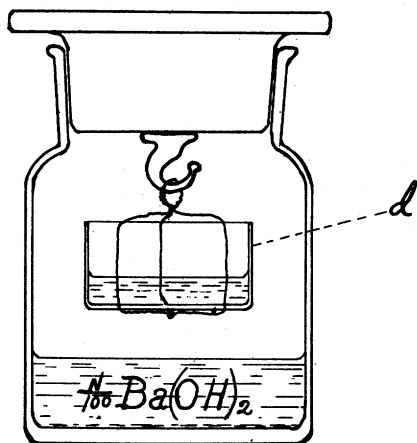


FIG. 1.

through a small hole in a cardboard disk which is placed over the mouth of the bottle shown in the photograph. The dish is then placed on the hook of the stopper and the bottle quickly stoppered as shown in Fig. 1. The ground glass stopper should be slightly greased and the bottle tightly stoppered. The CO_2 already in the air in the bottle and the CO_2 given off from the organisms in the suspended dish diffuses into the $\text{Ba}(\text{OH})_2$ solution and quickly forms the insoluble white precipitate of BaCO_3 . This absorption continues until no more $\text{Ba}(\text{OH})_2$ remains if sufficient CO_2 is produced to neutralize all the free base, for the BaCO_3 is practically insoluble and hence the reaction goes practically to completion. At the end of the desired period, the length of which is determined by the rate of CO_2 production by the organisms and conditions of the experiment, the stopper is quickly removed and the bottle is again covered with

the cardboard disk. A drop of phenolphthalein is added through the hole in the cardboard disk and then the $\text{Ba}(\text{OH})_2$ is rapidly titrated with a weak solution of HCl of known and appropriate concentration, say $n/100$. As control a similar bottle is set up in *exactly* the same way but *without* the animals. The $\text{Ba}(\text{OH})_2$ is titrated at the end of the experiment, that is, at the same time as the free $\text{Ba}(\text{OH})_2$ in the experimental bottle. The difference in the amounts of HCl necessary to neutralize the remaining free base in the bottles is equivalent to the quantity of CO_2 pro-

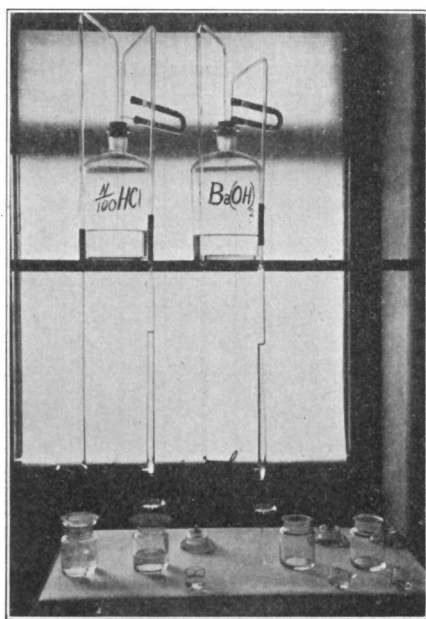


FIG. 2.

duced by the organisms during the period of the experiment. This description explains in brief the principle and procedure of the method, but the following sources of variation and error suggest themselves and will be considered in the same order as follows:

1. Temporary exposure to the CO_2 in the air of the open bottle containing the $\text{Ba}(\text{OH})_2$ may result in absorption of varying amounts of CO_2 in different individual bottles. This variation

may be due to non-uniformity of manipulation and different CO_2 content of the air in the room. The greatest source of error comes from the CO_2 in the expired air of the experimenter and from burning flames in the room.

2. If the organisms are in water in the dish, Fig. 1, *d*, then the CO_2 dissolved in this water either as dissolved free CO_2 or possibly partly in the form of bicarbonate, may vary unless precautions are taken to control this source of variation of CO_2 .

3. Another possible source of variation is the excretion of CO_2 by bacteria in the liquid in the dish, if this is for example a hay infusion containing *Paramecium* or from some other source depending upon the material experimented upon. This source of error is present and must be controlled in all other methods for CO_2 determination as well as in the one described here.

4. Finally perhaps the most important question is: how uniform, rapid and complete is the absorption of the CO_2 from the air in the bottle, by a $n/75$ to $n/150$ $\text{Ba}(\text{OH})_2$ solution?

Repeated tests have shown that all of the above four sources of error can readily be either eliminated or controlled if the following precautions are taken.

The first source of error mentioned under number one above can be controlled and thus practically eliminated by *working in a well-ventilated room in front of an open window through which fresh air from the out doors is entering*, and by *covering the mouth of the bottle with a perforated cardboard disk during the addition of the $\text{Ba}(\text{OH})_2$ at the beginning, and titration with HCl at the end of the experiment*. It is best to aërate the open bottle by holding or shaking it in the stream of air from the window just before covering it with cardboard disk and the rapid addition of the required amount of $\text{Ba}(\text{OH})_2$ from the burette. The pasteboard disk should be held tightly over the bottle during all the manipulation and only removed just before the stopper with the small dish is inserted. A similar procedure must be carried out at the end of the experiment during titration of the remaining free $\text{Ba}(\text{OH})_2$ with HCl .

The current of air from the window carries the exhaled air of the experimenter away from the bottles and makes the CO_2 content of the air the same in the duplicate bottles.

Sufficiently uniform manipulation in different determinations is very easily attained as the results below will show. The amount of CO_2 contained in the air in the bottle is determined by titrating with $n/100$ HCl , 15 c.c. of $n/100$ $\text{Ba}(\text{OH})_2$ in each of two bottles. One of these samples of $\text{Ba}(\text{OH})_2$ is titrated at once and the other at the end of an hour. The difference in the amounts of HCl is equivalent to the CO_2 of the air in each of the bottles at the beginning. To increase the accuracy three such tests are made at the same time and the average of the three taken as the true value.

The following are typical examples of tests showing the degree of uniformity.

Three samples of 15 c.c. $\text{Ba}(\text{OH})_2$ each when titrated at once using one drop of phenolphthalein took

| | |
|---------|---------------------------------|
| | 12.65 c.c. |
| | 12.70 c.c. |
| | <u>12.65 c.c.</u> |
| Average | 12.66 c.c. $n/100$ HCl |

At the end of 1 1/2 hours three other samples of 15 c.c. of the same $\text{Ba}(\text{OH})_2$ solution in each, took

| | |
|---------|-----------------------------------|
| | 12.35 c.c. |
| | 12.32 c.c. |
| | <u>12.32 c.c.</u> |
| Average | 12.33 c.c. $n/100$ HCl . |

The amount of CO_2 absorbed was therefore equivalent to the difference or .33 c.c. $n/100$ HCl .

Several days later another set of three samples of 10 c.c. each of $\text{Ba}(\text{OH})_2$ solution titrated at once, took

| | |
|---------|----------------------------------|
| | 8.32 c.c. |
| | 8.32 c.c. |
| | <u>8.35 c.c.</u> |
| Average | 8.33 c.c. $n/100$ HCl . |

At the end of 2 1/2 hours three corresponding samples took

| | |
|---------|-----------------------------|
| | 8.15 |
| | 8.13 |
| | <u>8.14</u> |
| Average | 8.14 $n/100$ HCl . |

The amount of CO_2 absorbed from the air in the bottle and during the manipulation on this day was therefore equivalent to .19 c.c. $n/100$ HCl .

The above illustrates the following facts: (a) The manipulation described above permits of uniform titration of the free $\text{Ba}(\text{OH})_2$. (b) The amounts of CO_2 absorbed in duplicate blank bottles after allowing time for absorption, are the same. (c) The CO_2 absorbed from the outdoor air during manipulation and from the volume of air enclosed in the bottle may vary from day to day with different conditions but is uniform over brief periods of time. It is therefore clear that whenever necessary, proper and adequate controls to determine this amount of CO_2 can readily be provided in experiments.

If the organisms are in water in the dish, Fig. 1, *d*, then another source of variation must be taken into account, viz., possible differences in amounts of CO_2 in the water in the dish. This may be made uniform by taking equal, and as small volumes as possible, of the medium in which the organisms live, and still further reduced to small and uniform amount by using tap water as a medium as for example with *Paramecium*, Lund ('18). That the variation exists and is quite definite is indicated in the following Table I.

TABLE I.

The native medium was tap water in which *Paramecia* had been living for several hours and from which they were removed with the centrifuge. Twenty c.c. $\text{Ba}(\text{OH})_2$ added to each bottle.

| Volume of Native Medium in each Dish. | | | |
|---------------------------------------|-------------------|-------------------|------------------------|
| 1 C.c. | 2 C.c. | 3 C.c. | None. |
| Titrated at End of 18 Hours. | | | Titrated at Beginning. |
| C.c. $n/100$ HCl. | C.c. $n/100$ HCl. | C.c. $n/100$ HCl. | C.c. $n/100$ HCl. |
| 20.45 | 20.20 | 20.00 | 21.40 |
| 20.61 | 20.35 | 19.97 | 21.35 |
| 20.55 | 20.30 | 20.00 | 21.40 |
| Average 20.53 | 20.26 | 19.99 | 21.36 |

With air breathing organisms this source of variation is of course absent. Escape of such organisms from the dish may be prevented by a wire gauze cap placed over the small dish.

In order to determine the rapidity and completeness of CO_2 absorption by the $\text{Ba}(\text{OH})_2$, simple and critical tests were carried

out as follows. A known amount, 1 to 5 milligrams of Na_2CO_3 were weighed out in a small glass vial. This vial with the carbonate was placed in 2 c.c.-3 c.c. 50 per cent. H_2SO_4 in the dish, Fig. 1, *d*, and suspended in the bottle over the $\text{Ba}(\text{OH})_2$. The vial was then tipped so as to allow the H_2SO_4 to decompose the carbonate, thus liberating all CO_2 from a weighed amount of Na_2CO_3 . The rate and completeness of absorption of this liberated CO_2 was then tested by titrating the $\text{Ba}(\text{OH})_2$ at different intervals of time after decomposition of the carbonate. A typical test was carried out as follows. Ten one milligram lots of Na_2CO_3 were weighed out in the glass vials as accurately as the balances and weights used would permit. One of the vials was placed in each bottle as described, over 10 c.c. $\text{Ba}(\text{OH})_2$ solution. The time when the Na_2CO_3 was decomposed was noted and titrations made at different intervals thereafter. Controls to determine the amount of CO_2 absorbed from the air in the bottles accompanied the experiment. Results of such an experiment are given in Table II.

TABLE II.

Lots of 1 milligram Na_2CO_3 each, decomposed with 50 per cent. H_2SO_4 in the closed bottle. Ten c.c. of an approximately $n/100$ $\text{Ba}(\text{OH})_2$ added to each bottle, Temperature 29°C .

| Bottle. | Controls. No Na_2CO_3 . Titrated with $n/100$ HCl at | | One Milligram Na_2CO_3 Decomposed. Titrated with $n/100$ HCl at End of | | | |
|--|---|------------------------------|---|-------------|---------|-----------------------|
| | Once. | End of $2\frac{1}{4}$ Hours. | 15 Minutes. | 30 Minutes. | 1 Hour. | $2\frac{1}{4}$ Hours. |
| 1 | 8.40 | 8.15 | 6.42 | 6.42 | 6.40 | 6.37 |
| 2 | 8.38 | 8.17 | 6.35 | 6.50 | 6.34 | 6.45 |
| 3 | 8.35 | 8.13 | 6.62 | 6.57 | | |
| Average | 8.38 | 8.15 | 6.46 | 6.49 | 6.37 | 6.41 |
| c.c. $n/100$ HCl equivalent of CO_2 in air | | .23 | | | | |
| Average | | | | | | |
| c.c. $n/100$ HCl equivalent of CO_2 re- covered from 1 mgm. Na_2CO_3 | | | 1.69 | 1.66 | 1.78 | 1.74 |
| c.c. $n/100$ HCl equivalent of CO_2 expected | | | | | | 1.84 |

The absorption was evidently very rapid and nearly complete in less than thirty minutes to an hour. The average amount of CO_2 not recovered in this experiment equivalent to about 0.1

c.c. .0098*n* HCl is perhaps largely due to error in weighing such a small quantity as one milligram on the balances which were only sensitive to about 1/20 mgm. Considering the small amounts of CO₂ involved, the degree of accuracy obtainable is rather striking; the amount of CO₂ apparently not recovered in the above test being about 5 per cent. of the CO₂ liberated from 1 mgm. Na₂CO₃ or about .01 c.c. CO₂ at N.T.P. In another similar experiment using lots of 1 mgm. Na₂CO₃ about 5 per cent. *more* CO₂ was recovered than was expected from 1 mgm. of carbonate. This indicates that the error was perhaps largely one due to weighing. In one test in which was used 2 mgm. of carbonate all of the CO₂ was absorbed in less than 1½ hours. Still another similar test to determine the rate of absorption of CO₂ from 5 mgm. of carbonate showed that at the end of fifteen minutes over 60 per cent.—70 per cent. of all the CO₂ was absorbed, and at the end of one hour all of the CO₂ was absorbed. To show that within the limits of error in weighing, the CO₂ in 2, 3 and 5 milligrams of carbonate can be practically completely recovered in relatively short periods of time by 15 c.c. weak solution of Ba(OH)₂ the results in Table III. are given.

TABLE III.

| Bottle. | Amount of Na ₂ CO ₃ Decomposed. | | |
|---|--|--|--|
| | 2 Mgms. | 3 Mgms. | 5 Mgms. |
| | Equivalent of CO ₂ Recovered, in C.c. .0098 <i>n</i> HCl. | Equivalent of CO ₂ Recovered, in C.c. .0098 <i>n</i> HCl. | Equivalent of CO ₂ Recovered, in C.c. .0098 <i>n</i> HCl. |
| 1 | 3.84 | 5.45 | 8.88 |
| 2 | 3.79 | 5.77 | 8.83 |
| 3 | 3.95 | 4.86 | 8.80 |
| 4 | 3.67 | 5.16 | 8.75 |
| 5 | 3.75 | 5.29 | |
| 6 | 3.39 | 5.05 | |
| Average | 3.73 | 5.26 | 8.81 |
| Average per milligram of Na ₂ - CO ₃ | 1.86 | 1.75 | 1.76 |
| Expected per milligram Na ₂ - CO ₃ | 1.84 | | |

The differences among the individual analyses were rather larger in this test than in some of the others. One and one half

to two hours were allowed for absorption of the CO_2 in the above test.

The applicability of the method to measurement of CO_2 , like some other methods depends upon the assumption that no other volatile acids than CO_2 are excreted by the organisms.

The carbon dioxide production by *Paramecium* and various small invertebrates has been measured and the results show that the method is satisfactory. For example in practical tests on *Paramecium* it was found that twice the number of *Paramecia* always produced twice the amount of CO_2 , other conditions being exactly the same in two experiments. When using *Paramecium* the method is of course brought to a most rigid test of its usefulness, since the amounts of CO_2 produced by 2 c.c.–4 c.c. of fairly concentrated *Paramecium* suspension in 8 to 24 hours are much smaller than those produced by an active animal weighing one gram. Success with the method depends to a considerable extent upon a proper appreciation of the rôle of the different factors described above and care in manipulation.

Among what appear to be the special advantages of the method are the following:

1. It is simple and direct.
2. Many determinations can be made in a short time when compared with other methods.
3. The range of adaptibility is considerable and the percentage of error of the method is relatively small when the small amounts of CO_2 which can be measured, is considered.
4. The apparatus is small and many bottles can be immersed in a water bath at the same time, hence temperature can be accurately controlled in many measurements carried out at the same time.

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